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A SEARCH INTO THE NITRATE AND NITRITE CONTENT OF WITTE'S "PEPTONE," WITH SPECIAL REFERENCE TO ITS INFLUENCE ON THE DEMONSTRATION OF THE INDOL AND CHOLERA RED REACTIONS.*

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IN a previous publication¹ I concluded, by rather rough experimental methods and deductions, that the cholera spirillum is not a nitrifying organism and that the successful demonstration of the cholera red reaction in a culture grown in a solution of Witte's "peptone" depends upon the reduction of a trace of nitrates. I was unaware at the time my experiments were performed that Petri² and Bleisch³ had already performed quite extensive and conclusive experiments upon this point. The latter worker also pointed out that the presence of an excess of nitrates or nitrites in the medium interfered with the reaction, and that the nitrate content of ordinary broth is so inconstant as to make it valueless for diagnostic purposes.

Some peculiar results in testing for indol, during the study of some bacteria from a case of irregular fever, emphasized for me the urgent necessity of adopting methods of preparing and testing media which would indicate the presence or absence of certain chemical constituents influencing such a biochemical test. This led to an investigation into the manner in which nitrates and nitrites may gain an entrance to media, and the influence of such variations on the demonstration of the indol and cholera red reactions.

It does not seem to me that the distinct difference between the indol and cholera red reactions has been sufficiently empha-

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¹ Bulletin No. 19, 1904, *Bureau of Government Laboratories, Manila, P. I.*

² *Centralbl. f. Bakt.*, 1889, 5, 561.

³ *Zeitschr. f. Hyg. u. Infektionskh.*, 1893, 14, 103.

sized in the past. By reference to the above-mentioned publication it will be seen that I did not fully appreciate this difference myself, for reasons which will be evident. Salkowski and Nencki¹ mistook the *purple* colored indol reactions for the cholera *red*, and although Petri and Bleisch undoubtedly produced the cholera red reaction, they do not dwell on the difference between it and the indol reaction. Kolle² says that "on adding small quantities of concentrated, chemically pure sulphuric or hydrochloric acid to bouillon or peptone cultures of cholera vibrios a violet or burgundy-wine-red color appears, as was shown by Poehl, and later by Bujwid and Dunham." Now there is a very distinct difference between the violet or purple colored indol reaction and the vermilion colored cholera red reaction when these tests are performed under carefully controlled conditions; and since both tests are of value in the differentiation of species, it seems advisable to define further the conditions under which they must be performed.

Further, my experiments have convinced me that the cholera spirillum does not produce nitrites in Dunham's peptone solution, made from Witte's "peptone" dialyzed free from nitrites, and that the apparent production of nitrites can be explained by the testing of uninoculated controls.

RESULTS OF A SEARCH FOR NITRATES AND NITRITES IN SOME INGREDIENTS USED IN PREPARING MEDIA.*

In testing for nitrogen, as nitrites, the naphthylamine hydrochloric and sulphanilic acid test was used in the manner usually employed in nesslerizing for ammonia, excepting that for purposes of comparison the reactions were performed in culture test tubes. In practice one c.c. of each test solution was added to 10 c.c. of the fluid to be tested. Where nitrates were sought for, the phenol sulphonic acid test was used and the reactions performed in three inch porcelain evaporating dishes.

The preliminary examination of a number of bottles of distilled water furnished by the Government Ice Plant showed that

¹ Vide the article by M. BLEISCH, *loc. cit.*

² KOLLE UND WASSERMAN, *Handbuch der Pathogenen Mikroorganismen*, 1903, 3, 21.

* I am indebted to Mr. C. L. Bliss, Physiological Chemist of the Bureau, for valuable advice during the course of the experiments.

this water sometimes contained nitrites in considerable quantities. So water was redistilled from potassium permanganate, collected in a perfectly clean fashion, and tested to prove the absence of nitrites and nitrates before use.

Several samples from a bottle of Kahlbaum's chemically pure sodium chlorid were found free from nitrates and nitrites. This was employed throughout the following experiments.

In testing "peptones" 0.1 gm. was dissolved in 10 c.c. of pure water, thus representing 10 c.c. of a one per cent peptone solution. In this way all our stock of Witte's "peptone," eight bottles, and in addition one bottle of Grüber's Pepton depur. sicc., were tested for nitrites. Some of the tests were repeated more than once, as indicated in Table 1, and in the last analysis, when the reactions were sufficiently marked, they were compared with the standard sodium nitrite colorimetric scale usually employed in the quantitative estimation of nitrites.

TABLE 1.

"Peptone"	No. of bottles	Nitrites (In 0.1 gm. "peptone") (In 10 c.c. water)	Nitrates (In 0.1 gm. "peptone")
Witte's Peptonum Siccum	1 {	(1) trace (2) 0.0000005 gm.N.	} 0
Witte's Peptonum Siccum	2 {	(1) 0 (2) 0 (3) 0	} 0
Pro Bacteriologie			
Peptonum Siccum	3 {	(1) distinct reaction (2) 0.0000005 gm.N.	} Not tested
Peptonum Siccum	4 {	(1) distinct reaction (2) trace	} Not tested
Peptonum Siccum	5 {	(1) trace (2) distinct reaction (3) 0	} 0
Pro Bacteriologie			
Peptonum Siccum	6 {	(1) trace (2) trace	} Not tested
Peptonum Siccum	7 {	(1) trace (2) distinct, but less than 0.0000005 gm.N.	} Not tested
Peptonum Siccum	8 {	(1) 0 (2) trace (3) trace	} Not tested
Pepton depur. sicc. Grüber	9 {	(1) trace (2) trace	} Not tested

These are the only records I have of a considerable number of such tests, but they suffice to show that nitrites occur in some

"peptones" in varying and often considerable quantities. They further show that nitrites may be distributed irregularly throughout the mass of "peptone," for when one sample, for instance, from the surface reacts to the test, another from a deeper portion of the bottle may be free from nitrites, or vice versa. This, together with further evidence to be presented, plainly indicates why the indol reaction may be obtained with one lot of media by the addition of chemically pure sulphuric acid alone, while with another the addition of nitrite is necessary.

All these "peptones" give a strong Biuret reaction, but only one was tested for the presence of peptones. Ten grammes from bottle No. 5 were dissolved in pure water and placed in a parchment dialyzer with all due precautions against introducing nitrites. After six hours at 28° the diffusate (about 150 c.c.) was tested. It did not give the Biuret reaction and 10 c.c. gave a reaction for nitrites equal to 0.0000005 gm.N.

Further, it was necessary to wash filter paper free from nitrites, e. g. a filter paper (Schleicher and Schülls) was washed with 200 c.c. nitrite-free water; 10 c.c. of the filtrate gave a reaction for nitrites corresponding to 0.0000005 gm.N.

The sulphuric acid used throughout these experiments was tested to prove the absence of nitrites.

EXPERIMENTS ON THE DEMONSTRATION OF THE INDOL AND CHOLERA RED REACTIONS IN DUNHAM'S PEPTONE SOLUTION.

In Undialyzed Peptone Solution.—Dunham's peptone solution, containing one per cent Witte's "peptone" (Bottle No. 5) and 0.5 per cent sodium chlorid, in redistilled water, was prepared with special precautions to avoid introducing nitrates and nitrites.*

Half of the medium was distributed in test tubes (10 c.c. per tube) and marked "peptone solution only." To the other half was added 0.01 per cent of a freshly prepared solution of sodium nitrate and this was then distributed in a similar manner. Both sets were autoclaved for half an hour at 120°. Final reaction, 0.5

*A large glass beaker was found to be especially convenient for boiling medium, as one can more easily mark the initial height of the latter and watch the completeness of solution, than when the cooking is performed in an agate-ware boiler. The albumoses do not all go into solution in the presence of 0.5 per cent sodium chlorid.

per cent acid to phenolphthalein. A tube of each of the media tested for nitrites gave a reaction equal to about 0.0000005 gm.N.

(a) One tube of each medium was inoculated with cholera 579.* After 18 hours' growth at 36° to 37°, 0.5 c.c. of pure sulphuric acid was added to each culture. The culture marked "peptone solution only" yielded an immediate *purple*-colored indol reaction. The culture marked "peptone solution only + 0.01 per cent NaNO₃" did not give an immediate reaction, but in about half an hour developed an intense *red* color (cholera red reaction).

(b) That this "peptone solution only" is eminently suitable for the production of indol by *B. coli* 577† is shown by the following experiment.

Three tubes were inoculated each day for eight successive days and the cultures kept at 36° to 37°. Bearing in mind the observation of A. W. Peckham¹, that with different cultures (*coli*) the maximum amount of indol could be decided only by corresponding variations in the proportions of nitrites and acid used, I experimented to see whether a similar variation in the amount of nitrites and acid was necessary to elicit the maximum indol reaction in cultures in the same organism, for example, of two and eight days' growth.

To each series of three tubes, then, one-half, one and two c.c. of a 0.01 per cent sodium nitrite solution and corresponding amounts of pure sulphuric acid were added. Throughout the series the most intense reaction was elicited by using 0.5 c.c. of the test solutions, with the exception of the seven-day old series, where 1 c.c. yielded the best reaction. The c.c. invariably yielded enough nitrous acid to obscure the reaction. It may be of interest to plot the gradual increase of indol as shown in Chart 1, where the reactions of greatest intensity are compared with the colorimetric scale of standard sodium nitrite dilutions.

Throughout the following experiments the test for indol was

* Unfortunately I have been unable to test all the cholera cultures described in Bulletin 19 of this Bureau, owing to the accidental death of nearly all my stock cultures. This strain of culture, 579, was reclaimed by plating Dr. W. E. Musgrave's ameba culture 11524 grown in pure symbiosis with this spirillum.

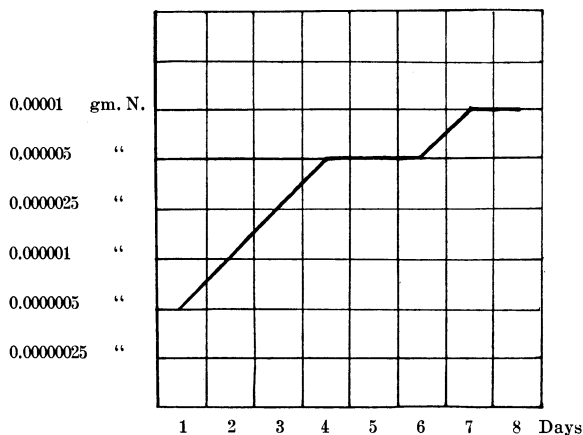
† A culture kept in stock for two years, the fermentation tests of which are given on p. 24, Bulletin 19, Bureau of Government Laboratories, Manila, P. I.

¹ *Jour. Exper. Med.*, 1897, 2, 560.

made by adding 0.5 c.c. of a freshly prepared 0.01 per cent solution of sodium nitrite and 0.5 c.c. of pure sulphuric acid. Cholera red was tested for by adding 0.5 c.c. of pure sulphuric acid.

(c) Such an albuminous medium, moreover, seems to absorb nitrites from the laboratory atmosphere much more rapidly than distilled water does, for this "peptone" solution, which at first gave reactions for nitrites corresponding to about 0.0000005 gm. N., after two weeks at 18° to 28° gave reactions corresponding to

CHART 1.



about 0.0000025 gm. N. an increase quite sufficient to yield an indol reaction when pure sulphuric acid alone is added to a 24-hour culture of *B. coli* 577.

This increase of nitrites was not due to an increase in the concentration of the medium, for the initial level of the medium had been marked on each tube and very little evaporation had taken place. Further, this increase in the amount of nitrites in the medium containing nitrates is sufficient to obscure somewhat the bright vermilion red color of the cholera red reaction.

In Dialyzed Peptone Solution (Nitrate- and Nitrite-Free).—As there was very little "peptone" left in bottle No. 2, 10 grammes from bottle No. 5 were dissolved by boiling in about 200 c.c. of pure water. It was then dialyzed through parchment with due precautions against introducing nitrates or nitrites or any other

foreign substance. As mentioned above, no peptones were found in the first diffusate (six hour), which gave a reaction for nitrites. The process was continued for 24 hours during two days, the contents of the dialyzer being removed, concentrated by boiling and sterilized at necessary intervals. At this time 10 c.c. of the contents of the dialyzer gave no reaction for nitrites. Dunham's peptone solution was then made up as in the experiments with undialyzed "peptone"—one set being marked "nitrite-free peptone" and the other "nitrite-free peptone + 0.01 per cent NaNO_3 ." After autoclaving, the "nitrite-free peptone" solution gave no reaction for nitrites or nitrates, while the other gave no reaction for nitrites but quite a distinct reaction for nitrates. The final reaction of this dialyzed medium was 0.1 per cent alkaline to phenolphthalein, showing the removal of substances capable of reacting acid to phenolphthalein to the extent of 0.6 per cent.

A series of tubes inoculated with cholera 579 was tested after 17 hours at 36° to 37° , with the following results:

TABLE 2.

Medium	Culture	Hours at 36° to 37°	Nitrites	Cholera Red	Indol	Nitrites in Uninoculated Controls
"Nitrite-free peptone"	579	17	—	—	+ Deep purple color immediately	—
"Nitrate-free peptone" + 0.1% NaNO_3	579	17	Intense Reaction	+ Light red color after several hrs. 36° to 37°		—

In about 10 days however, this dialyzed medium had absorbed enough nitrites to give a distinct reaction, and without the use of uninoculated controls one might have assumed that the nitrites were produced through the activity of the cholera spirillum, as may be seen in Table 3.

It will also be noted that the faint trace of nitrites absorbed from the atmosphere was not sufficient to yield any reaction, when sulphuric acid alone was added, but that the addition of nitrite and sulphuric acid produced a very distinct indol reaction.

TABLE 3.

Medium	Culture	Days at 36° to 37°	Nitrites	Nitrites in Uninoculated Controls	Cholera Red	Indol
"Nitrite-free peptone"	Cholera 579	1	Faint reaction	Faint reaction equal to that in tubes inoculated with cholera 579 and of equal intensity when compared with each other	—	+ Deep purple color
"	"	2	All three of equal intensity		—	"
"	"	3			—	"
"	Coli 577	1				—
"	"	2				+ (Faint)
"	"	3				+ (marked reaction)

In the dialyzed medium containing 0.01 per cent sodium nitrate, the cholera spirillum almost completely reduces the nitrates to nitrites in 24 hours, as shown in Table 4.

TABLE 4.

Medium	Culture	Days at 36° to 37°	Nitrites	Nitrates	Cholera Red	Indol
Nitrite-free peptone +0.01% NaNO ₃	Cholera 579	1	Marked reaction	Faint reaction	Yellowish	Yellowish
"	"	2		Negative (Not tested)	Reddish-yellow Distinct and marked red color with none of the purple color of indol reaction	Reddish-yellow Distinct and marked red color with none of the purple color of indol reaction
"	"	3				
"	Coli 577	1			As per cholera 579, but of lesser intensity	
"	"	2				
"	"	3				

Here an immediate and distinct cholera red reaction was not obtained until the organism had grown for three days at 36° to 37°. This experiment (which is one of a number) also shows that, when growth takes place in the presence of 0.01 per cent sodium nitrate, even if a trace of nitrite be also originally present, something (an oxidation product of indol?) is produced which is demonstrable as "cholera red,"* whether sulphuric acid alone or

*According to Kolle (*loc. cit.*), "cholera red" was isolated in a pure state by Brieger, who considered the cholera red reaction as nothing more than the nitrose-indol reaction, the nitrous acid set free combining with the indol to produce a new substance, the cholera red. Brieger's original articles (*Deutsche med. Wchnschr.* 1887, 13, p. 303) are not within reach, so I cannot discuss this point.

sulphuric acid and nitrite are added subsequently, though such a further addition of nitrite may obscure the reaction.

On the other hand, a few days' growth in the presence of sufficient nitrite to yield the indol reaction does not influence the character of the indol formed; but if a considerable amount of nitrite be present in the medium, the further addition of nitrite yields enough nitrous acid to obscure the indol reaction.

SUMMARY AND CONCLUSIONS.

1. Nitrites, and probably nitrates also, may gain entrance to artificial media from various sources—certain waters, “peptones,” and filter papers—yielding distinct reactions for nitrites with naphthylamine hydrochlorid and sulphanilic acid.

2. A sufficient quantity of nitrites may be further absorbed, in a few days, from the laboratory atmosphere, to yield a distinct *indol* reaction upon the addition of 0.5 c.c. of chemically pure sulphuric acid to, for example, a culture of *B. coli* 577 grown for 24 hours at 36° to 37° in 10 c.c. of Dunham's peptone solution, which originally contained insufficient nitrite to yield such a reaction.

3. The cholera spirillum does not produce nitrites in nitrate- and nitrite-free “peptone” solution, prepared from Witte's “peptone” dialyzed free from nitrites; and when nitrites are apparently formed, their presence may be explained by the simultaneous testing of uninoculated controls. In this I disagree with Bleisch, who believed that small quantities of nitrites could be formed, and agree with Petri, who considered the existence of such a nitrifying power as unproved as well as improbable.

4. The vermilion-colored “cholera red reaction” must be distinguished from the purple-colored “indol reaction.”

5. The production of “cholera red,” by the cholera spirillum or *B. coli*, takes place only, under the conditions of these experiments, during the reduction of a trace of nitrates, and when formed is demonstrable as such, whether pure sulphuric acid alone or sulphuric acid and nitrite are added, although an excess of nitrite furnishes enough nitrous acid to obscure the reaction.

6. The growth of *B. coli* or the cholera spirillum in the presence of sufficient nitrite to yield the indol reaction on the addition of a pure acid alone does not influence the character of the indol formed; but if a considerable amount of nitrite be present in the medium, the further addition of nitrite yields enough nitrous acid to obscure the indol reaction.

7. The cholera red reaction is not specific.

8. It is recommended that media used in testing for the production of indol or cholera red be examined for nitrates and nitrites before use.